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Short Communication

Inactivation of Normal and Distorted DNA-Binding Proteins as a Potential Indicator of High Hydrostatic Pressure-Induced Lethal Injury in Foodborne Pathogens

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Abstract

Background: Our previous studies observed bacterial growth in phosphate buffer and refrigerated seafood after treatment with high hydrostatic pressure (HHP) at 300 megapascals (MPa), but not at 500 MPa, reflecting the sublethal and lethal effects of 300 and 500 MPa, respectively. Thus, this study hypothesized that whether HHP induces a sublethal or a lethal effect on foodborne microorganisms, which is primarily determined by the ability of HHP at 500 MPa to inactivate proteins that interact with normal and distorted DNA, based on the importance of protein–DNA interactions in cell division and the ability of HHP to distort DNA. **Methods:** An electrophoretic mobility shift assay (EMSA) was conducted to examine the impact of HHP on DNA–binding protein activity in three bacterial strains commonly found in seafood, with binding activity in strains grown at 0.1 MPa used as a positive control. After collecting the growing bacterial cells by centrifugation, the pellets were resuspended in saline and treated with HHP. Extracts of HHP-treated or untreated *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Vibrio parahaemolyticus* were incubated with a duplex oligonucleotide containing either a normal helical structure or a structure twisted by ultraviolet (UV)-induced (6-4) photoproduct (6-4PP). Band intensities of protein–DNA binding complexes were quantified by image analysis. **Results:** Detectable levels of protein activity interacting with normal or 6-4PP-twisted DNA were found in extracts from all three strains. HHP at 300 MPa exerted weak inhibitory effects on bacterial distorted DNA-binding activity. However, HHP at 500 MPa inactivated both normal and distorted DNA-binding activities across all three strains, reducing activities to 0–16% of control values. In contrast, HHP at 500 MPa exerted variable inhibitory effects on Na^+/K^+-ATPase and catalase activities across the different strains. **Conclusions:** Normal and distorted DNA-binding activities in the three representative strains were inactivated when HHP was increased from 300 to 500 MPa, indicating the high sensitivity of bacterial DNA-binding factors to HHP. Since the loss of DNA-binding capacity strongly correlated with HHP-induced bacterial killing observed in our previous studies, the inactivation of normal and distorted DNA-binding factors may serve as a potential indicator of HHP-induced lethal effects in foodborne pathogens.

Keywords: bacteria; biomarker; DNA; high hydrostatic pressure; lethal injuries; UV

1. Introduction

High hydrostatic pressure (HHP) is a nonthermal technology that kills foodborne pathogens in aquatic products, beverages, fruit, meat products and vegetable products [1,2,3]. Due to the ability of pressures at several hundred megapascal (MPa) to damage microbial macromolecules at ambient or chilled temperatures, HHP eradicates microorganisms while minimizes the loss of color, flavor, and nutrients [4,5]. Previous studies indicated protein denaturation as the primary mechanism of HHP-induced inhibition of microbial growth [6,7,8]. Disappearance of many outer membrane proteins was found by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after treating *Salmonella Typhimurium*, a leading microbial strain of foodborne diseases, with HHP at 350–600 MPa under 20 ℃ for 10 min [7]. Morphological lesions, including breakdown of the peptidoglycan layer, partial loss of the cell envelope and rupture of the cell wall, were observed by both scanning and transmission electron microscope when *Vibrio parahaemolyticus*, a strain commonly found in seafoods, was exposed to HHP at 300 MPa for 10 min at room temperature [8].

In addition to its damaging effects on membrane structure and protein conformation, HHP also disrupts the integrity of chromosomal DNA in microorganisms. Pulsed-field gel electrophoresis (PFGE) conducted in an earlier investigation demonstrated the capability of HHP at 530 MPa for 30 min to cause chromosomal DNA breakage in *Shigella dysenteriae* or *Escherichia coli* resuspended in cold raw milk [9]. HHP at 200 MPa was also reported to distort the B-form helical structure of a synthetic oligonucleotide via a widening of the minor groove according to nuclear magnetic resonance (NMR) spectroscopy, evidencing a direct influence of HHP on DNA conformation [10].

Helix-distorting DNA lesions in living organisms are primarily removed by nucleotide excision repair (NER) [11] and UvrABC endonuclease is the NER system operating in bacteria with $UvrA_2B$ complex as the damage recognition molecule [12].

Despite its extensive use for food preservation, survival of pathogenic microorganisms in HHP-treated foods because of sublethal injuries has been considered as a major risk associated with the application of this technology [2,13]. Microorganisms suffering from sublethal injuries may begin to proliferate once the damaged cellular components have been repaired [14] through mechanisms such as protein refolding [15]. Our earlier studies revealed differential sensitivity of foodborne microorganisms to increasing levels of HHP [16,17]. The application of a pressure at 400–500 MPa for 2 min at 25 ℃ was found to eliminate seafood-associated histamine-forming bacteria *Morganella morganii* and *Photobacterium phosphoreum* suspended in phosphate buffer, while surviving cells were apparently observed after treating both strains with HHP at 300 MPa for 6 min [16] (**Supplementary Fig. 1**). Aerobic plate count and psychrotrophic bacterial count showed the ability of HHP at 500 MPa, but not at 300 MPa, to block a partial recovery of microbial growth in frozen soy sauce-marinated freshwater clam samples [17] (**Supplementary Fig. 2**). Hence, 500 MPa kills disease-causing bacteria present both in a simple buffer and frozen seafoods and the sublethal effect of 300 MPa might result from its inability to block repair machineries.

Because of the diversity of microbial taxonomic units in nature and the significant different responses of proteins in one bacterial strain to the same HHP level [7,8], it is important to explore critical protein targets which may serve as biomarkers to discriminate between HHP-induced lethal and sublethal effects. As DNA binding proteins involved in replication and gene expression along with those targeting distorted DNA during the damage recognition stage of NER are indispensable for cell growth and survival, it is very likely that whether HHP induces a sublethal or a lethal effect on foodborne microorganisms is determined by the ability of this HHP level to destroy proteins interacting with normal and distorted helical structures. Therefore, this study examined if there existed a good correlation between inactivation of DNA binding protein activities and HHP-induced lethal effects on foodborne pathogens.

UV irradiation on DNA induces the formation of two major types of helix-twisting lesions identified as cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (6-4PP) [18]. Compared to a 9° helix distortion generated by CPD, 6-4PP induces a severe bending of DNA at 44° [19] and 6-4PP has been shown to be a better substrate for NER proteins in both prokaryotes [20] and eukaryotes [21], making inactivation of 6-4PP detecting proteins a good indicator for loss of NER capacity. Hence, a UV-irradiated duplex oligonucleotide containing a 6-4PP was used as a representative of twisted DNA in our electrophoretic mobility shift assay (EMSA), whereas unirradiated oligonucleotide was regarded as DNA with normal conformation. The extracts of HHP-treated and untreated Gram-negative *Vibrio parahaemolyticus*, *Salmonella Typhimurium*, and Gram-positive *Listeria monocytogenes* were used as the sources of DNA binding proteins since these strains were found in our freshwater clam samples [17].

2. Materials and Methods

2.1 Bacterial Culture Condition

Listeria monocytogenes (BCRC15338), *Salmonella Typhimurium* (BCRC12947) and *Vibrio parahaemolyticus* (BCRC13023) in freeze-dried powder form were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). Bacteria were resuscitated by incubation at 37 °C for 16–18 h in 10 mL sterile tryptic soy broth (TSB) (Sigma Aldrich, France) followed by streaking on tryptic soy agar plates. For *V. parahaemolyticus*, the culture media were additionally supplemented by 2.5% NaCl. An isolated colony was then loop transferred from each plate into a tube containing 5 mL TSB and tubes were kept at 37 °C overnight under shaking. Subcultures were prepared by transferring 1-mL portions of the precultures to 200 mL fresh TSB and incubated at 37 °C for 24 h. Bacterial pellets were collected from 100 mL aliquots of the subcultures by centrifugation at 4000 ×g at 4 °C for 10 min and washed twice with sterile saline (0.85% NaCl) followed by centrifugation. The sedimented cells were resuspended in saline and cell concentration was adjusted to approximately 10^7 CFU/mL.

2.2 HHP Treatment

Bacterial cell suspensions aseptically transferred to sterile polyethylene bags in 8-mL portions were vacuum packaged and heat sealed immediately. Sealed bags containing cell suspensions were vacuum packaged in new bags to prevent bag leakage during HHP treatment. HHP treatment was performed using an industrial scale high-pressure processing system with a 6.2-L vessel (Bao Tou Ke Fa, China) that operates at pressures up to 600 MPa. Water at 20 °C was used as the transmission fluid for pressure in this study and hydrostatic pressure was increased at a rate of 45 MPa/s and released within 10–15 s. Triplicate vacuum bags containing bacteria suspended in saline were subjected to pressure at 300 or 500 MPa in triplicate, with a holding time of 3 min at room temperature. After HHP treatment, samples were refrigerated at 4 °C for 24 h until further processing. Portions of cell suspensions were transferred to bags followed by vacuum package and refrigerated under atmospheric pressure (0.1 MPa) to serve as untreated controls.

2.3 Preparation of Bacterial Extracts

Refrigerated bacteria in bags were harvested by centrifuging at 16,000 ×g for 10 min at 4 °C and supernatants that might contain leaked proteins were also collected. Cell pellets were then lysed in cold RIPA lysis buffer (Energenesis Biomedical Co. Ltd. Taipei, Taiwan) under vigorous vortexing. After centrifugation at 22,000 ×g for 10 min at 4 °C, cell lysates were combined with supernatants and bacterial extracts were obtained by filtering the combined solutions through a 0.22 µm pore size Millex-GS syringe filter (Millipore, Burlington, MA, USA). The protein concentration of each sample was determined using a BCA protein assay kit (catalog number 23225, Thermo Fisher Scientific, Waltham, MA, USA).

2.4 Preparation of Duplex Oligonucleotides as Targets for DNA Binding Proteins

A 26-mer oligonucleotide 5′-AGG CAT GCA TGC ATC GCG CGC GTA TA-3′ (TC probe) employed by a previous investigation [22] was synthesized and biotin-labeled at the 5′ end by a commercial source (Bertec, Taipei, Taiwan). A duplex oligonucleotide representing DNA with a normal helical structure was prepared by annealing biotin-labeled TC probe with its biotin-labeled complementary strand. A duplex oligonucleotide representing twisted DNA was produced by irradiating the annealed probe (6 pmol in 2 µL TE buffer) on a piece of parafilm with UV (254 nm) at an intensity of 9 kJ/m^2 in a UV crosslinker (XL-1500, Spectronics, Melville, NY, USA). UV irradiation at an energy level of 9 kJ/m^2 has been shown to generate a helix-distorting (6-4) photoproduct (6-4PP) between the two adjacent TC sites underlined according to chemical analysis [22] and our protein binding assay [21].

2.5 Electrophoretic Mobility Shift Assay (EMSA)

UV-irradiated or unirradiated TC probe (6 pmol) was mixed with bacterial extracts containing 5 µg proteins in a 20-µL mixture on ice in the presence of 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, and 2.5 µg poly dI-dC. Poly dI-dC was included in EMSA mixtures as a DNA competitor to reduce nonspecific protein-DNA interactions [23]. After mixing all components, the reaction mixture was incubated at 30 °C for 20 min and electrophoresed on a 8% nondenaturing polyacrylamide gel to separate protein-bound and free probe. All components on the gel were blotted onto a nylon membrane and crosslinked by UV (254 nm) irradiation at 1200 J/m^2. The membrane was subsequently blocked with 5% (w/v) skimmed milk and the presence of protein-DNA binding complexes was detected by further incubation of the membrane with streptavidin-conjugated alkaline phosphatase and chemiluminescent enzyme substrates. After exposing to X-ray films, band shift intensities were determined using an image analysis software (Multi Gauge version 3.0, Fuji Film, Tokyo, Japan).

2.6 Effects of HHP on Na^+/K^+-ATPase and Catalase Enzyme Activities

Na^+/K^+-ATPase regulates ion transport on cell membranes and catalase plays an important role in the antioxidant system because of its ability to remove cell-damaging hydrogen peroxide. As these two enzymes are critical to cell survival, the responses of Na^+/K^+-ATPase and catalase to HHP were determined in comparison with those of DNA binding factors. After collecting bacterial pellets by centrifugation, cells were lysed and enzyme activities in cell lysates were measured according to the protocols described in Chekine^{TM} Micro Na^+/K^+-ATPase and catalase activity assay kits (Abbkine Inc., Wuhan, China).

2.7 Statistical Analysis

Band intensities of protein-DNA binding complexes obtained from EMSA (n = 4) and enzyme activities measured in bacterial extracts (n = 3) were expressed as the mean and standard deviation (S.D.). A normal distribution of data was first verified by Shapiro-Wilk test and homogeneity of variance was confirmed by the Levene test. Statistical differences were determined using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison method. A probability of $p < 0.05$ indicates statistically significant. The strengths of statistical differences were assessed by effect sizes using Cohen's d with values >0.8 indicate strong statistical differences. All statistical differences shown in this study are accompanied by d values >1.8.

3. Results

3.1 Effects of HHP on Normal and Twisted DNA Binding Activities in L. monocytogenes, S. Typhimurium and V. parahaemolyticus

The extracts of *L. monocytogenes* grown under 0.1 MPa interacted with the duplex oligonucleotide containing a normal or a 6-4PP-twisted helical structure to form two protein-DNA binding complexes (band shifts A and B) with apparently different electrophoretic mobilities (Fig. 1A). The intensity of band shift A was frequently found to be a little stronger when extract proteins were incubated with 6-4PP-twisted probe, suggesting the high affinity of some proteins for distorted DNA (Fig. 1A). HHP at 300 MPa selectively repressed the formation of band shift B regardless of the probe type. Both normal and distorted DNA binding proteins activities in *L. monocytogenes* were almost eliminated by HHP at 500 MPa as HHP-treated *L. monocytogenes* extracts generated only band shift A at barely detectable levels (Fig. 1A). Incubation of the extracts of untreated *S. Typhimurium* with normal or distorted DNA generated only one band shift and a stronger binding of extract proteins to distorted DNA was also observed (Fig. 1B). Distorted DNA binding activities in *S. Typhimurium* were weakly inhibited by HHP at 300 MPa. HHP at 500 MPa strongly suppressed normal and twisted DNA bind-

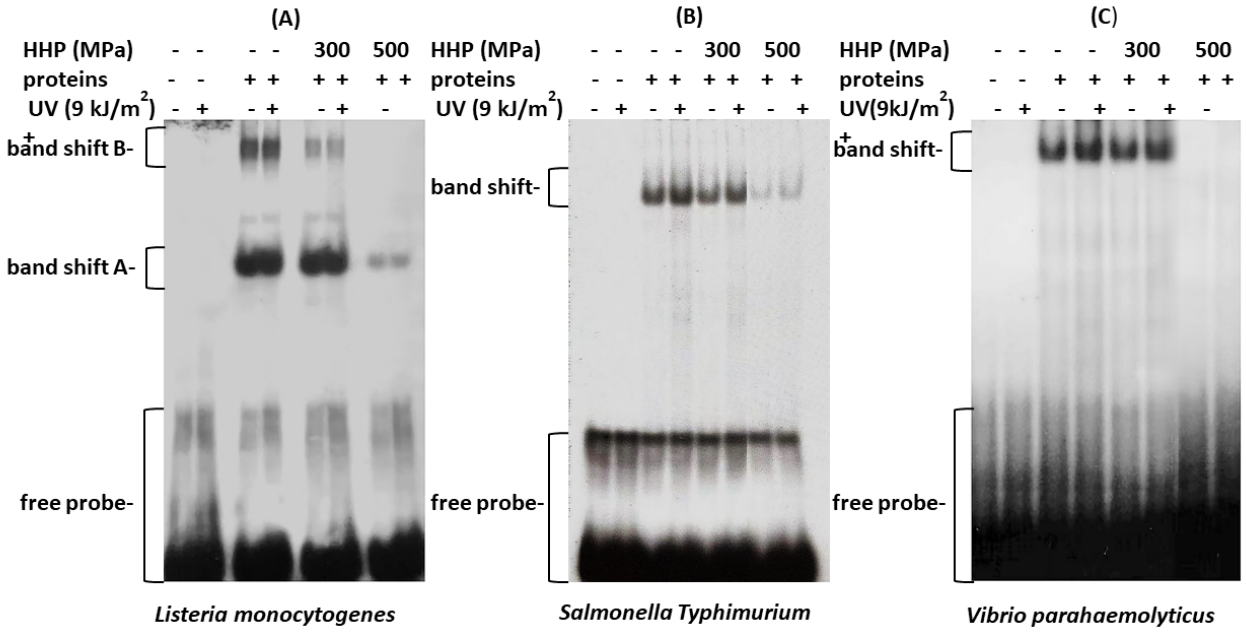

Fig. 1. Effects of increasing levels of high hydrostatic pressure (HHP) on DNA binding activities in (A) *L. monocytogenes* (B) *S. Typhimurium* and (C) *V. parahaemolyticus*. Bacterial extracts were prepared from cells grown under 0.1 megapascals (MPa) (control) and from cells subjected to HHP at 300 or 500 MPa at room temperature. A duplex oligonucleotide carrying normal or (6-4) photoproducts (6-4PP)-distorted helical structure was incubated with 5 μg extract proteins and the influence of HHP on protein-DNA interactions was examined by electrophoretic mobility shift assay (EMSA). Band shifts A and B indicate protein-DNA binding complexes with different electrophoretic mobilities. FP stands for free probe. This figure is representative of four separate experiments.

ing activities in *S. Typhimurium* to barely observable levels (Fig. 1B). The extracts of untreated *V. parahaemolyticus* also produced one band shift when mixed with either type of probe and these extracts produced stronger 6-4PP-dependent band shift (Fig. 1C). While HHP at 300 MPa weakly inhibited distorted DNA binding activities, HHP at 500 MPa fully eliminated the activities of normal and distorted DNA binding molecules in *V. parahaemolyticus* (Fig. 1C).

3.2 Quantitative Analysis of Band Shift Experiments

Using normal and distorted DNA binding activities in each strain cultured under 0.1 MPa as positive controls, quantitative analysis of band shift formation indicated that HHP at 300 MPa downregulated normal DNA binding activities in *L. monocytogenes* to 70% of the control value. However, this pressure did not affect normal DNA binding capacity in *S. Typhimurium* and *V. parahaemolyticus* (Fig. 2A). Normal DNA binding activities in different strains were very sensitive to 500 MPa as these activities were suppressed to 0 to 16% of control after HHP treatment (Fig. 2A). HHP at 300 MPa weakly inhibited distorted DNA binding activities in all three strains (Fig. 2B). In agreement with its common effect on normal DNA binding capacity, HHP at 500 MPa either eliminated or strongly suppressed distorted DNA binding activities to barely detectable levels (Fig. 2B).

3.3 Impact of HHP on Bacterial Na^+/K^+-ATPase and Catalase Activities

HHP at 300 MPa already induced a significant inhibition of Na^+/K^+-ATPase activity in *L. monocytogenes* and *S. Typhimurium* and HHP at 500 MPa further repressed the catalytic power of this enzyme. Although Na^+/K^+-ATPase activity in *V. parahaemolyticus* was inhibited by 300 MPa, this activity was unresponsive to pressure increase from 300 to 500 MPa (Fig. 3A). Activity measurement indicated the susceptibility of catalase in all three strains to 300 MPa and 500 MPa imposed much stronger inhibitory effects on enzyme activities in *L. monocytogenes* and *V. parahaemolyticus*, yet this pressure failed to further repress catalase activity in *S. Typhimurium* (Fig. 3B). In contrast to the sharp reduction of normal and distorted DNA binding activities in all three strains after raising HHP from 300 to 500 MPa, enzyme activity assays revealed no consistent responses of bacterial Na^+/K^+ -ATPase and catalase to increasing levels of HHP.

4. Discussion

Based on its simplicity in performance, EMSA using tracer-labeled synthetic oligonucleotides as protein binding targets is regarded as a rapid and sensitive method to detect the interactions between protein and DNA or RNA [23]. Our EMSA detected apparent levels of normal and twisted

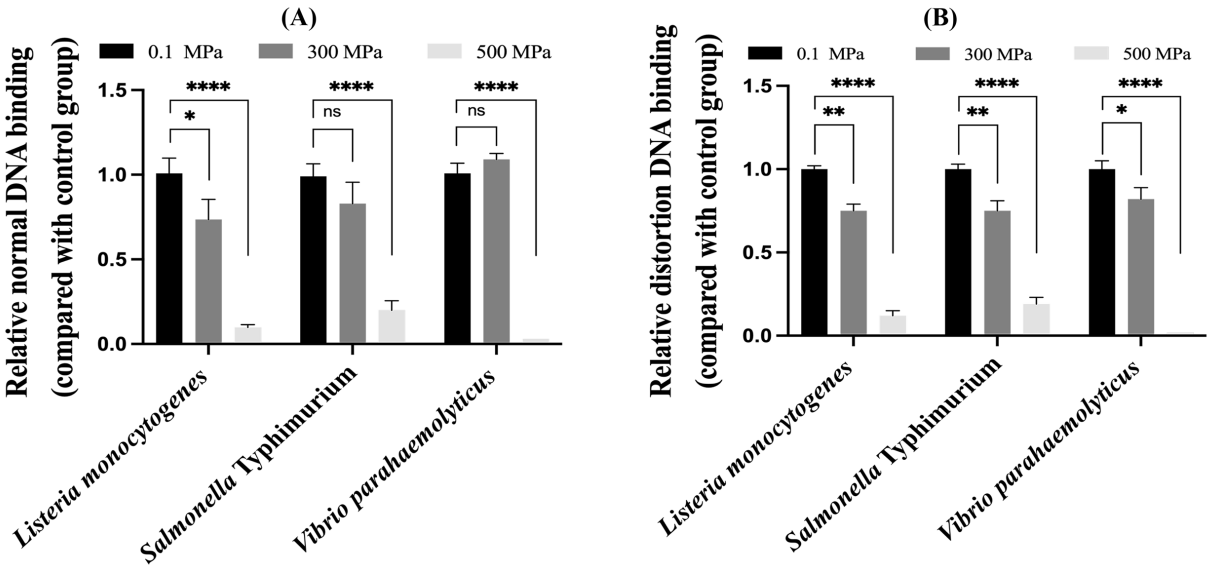

Fig. 2. Quantitative analysis of the effects of HHP on (A) normal and (B) distorted DNA binding protein activities in *L. monocytogenes*, *S. Typhimurium* and *V. parahaemolyticus*. DNA binding activities in strains cultured under 0.1 MPa were used as positive controls. Each bar graph represents the mean and standard deviation of four separate experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$ and **** indicates $p < 0.001$ when compared to control. ns indicates no statistical significance.

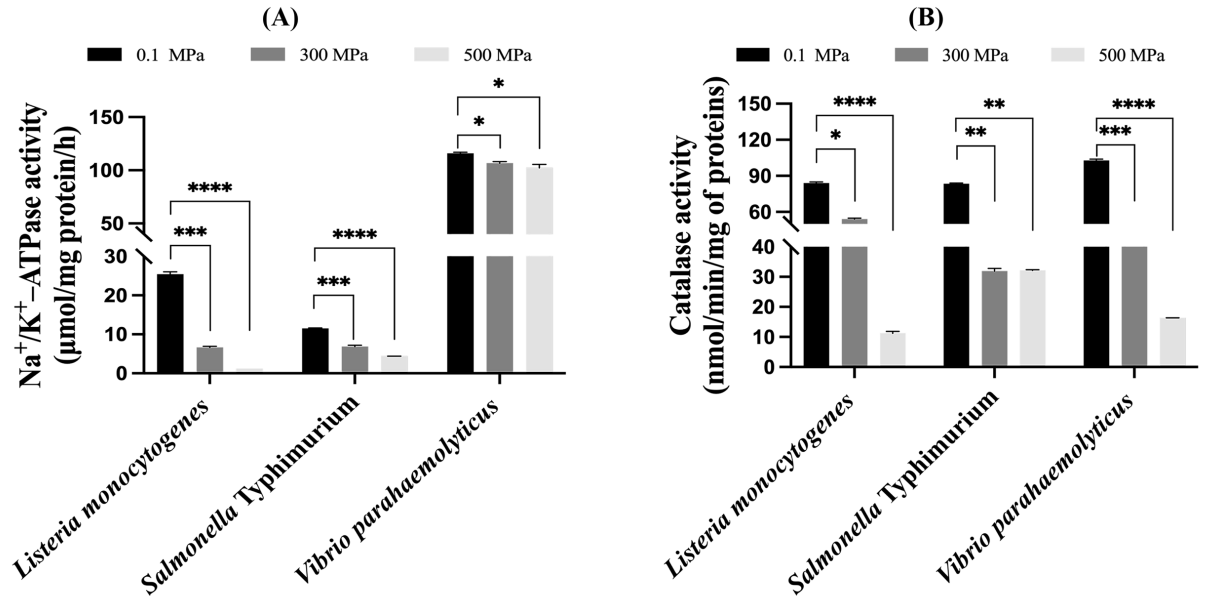

Fig. 3. Varied responses of (A) Na^+/K^+-ATPase and (B) catalase activities in different bacterial strains to HHP. Enzyme activities in the extracts of control and HHP-treated strains were measured by assay kits from a commercial supplier. Na^+/K^+-ATPase and catalase activities were expressed as μmol/mg protein/h and nmol/mg protein/min, respectively. Each bar graph represents the mean and standard deviation of three separate experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.005$ and **** indicates $p < 0.001$ when compared to control.

DNA binding protein activities in the extracts of control *L. monocytogenes*, *S. Typhimurium* and *V. parahaemolyticus* cultured under 0.1 MPa. Incubation of bacterial extracts with 6-4PP-distorted probe usually produced protein-DNA complexes with a little stronger band intensity.

It is well known that the components of UvrABC endonuclease that sense and excise helix-distorting DNA lesions are constitutively expressed in scarce amounts in *Escherichia coli*, making it very difficult to purify these factors. In the absence of DNA damage, it is likely that

UvrABC proteins were also expressed at low levels in bacterial strains used in this study, thus the amount of UvrABC molecules present in bacterial extracts added to EMSA mixtures was too low to accomplish a strong interaction with 6-4PP-distorted DNA.

When single-stranded DNA is accumulated in *E. coli* due to blockage of DNA replication induced by DNA damaging agents such as UV, a response called SOS response is activated to relieve the low expression of SOS genes, including those encoding UvrA and UvrB, controlled by a transcriptional repressor LexA. Therefore, DNA repair and cell survival is promoted by upregulated production of Uvr proteins [24]. Failure of HHP at 300 MPa to upregulate distorted DNA binding activities in different strains implied that no or very weak SOS response was induced by this pressure.

The weak impact of 300 MPa on DNA binding protein activities in different strains strongly suggested that a large proportion of proteins associated with gene activation remained available under 300 MPa and these proteins might regulate the production of molecules capable of repairing pressure-damaged cell components and finally led to a partial recovery of bacterial growth as found in our previous studies on the survival rates of bacteria in phosphate buffer and frozen soy sauce-marinated freshwater clam samples following HHP treatment [16,17].

Very different from the effects of 300 MPa on DNA binding capacity, HHP at 500 MPa inactivated both normal and distorted DNA binding protein activities in all three strains, indicating the ability of 500 MPa to cause a breakdown of DNA binding protein structure. SOS response also seemed to be absent in bacterial strains treated with HHP at 500 MPa. The drastic reduction of normal and distorted DNA binding protein activities in response to elevation of HHP from 300 to 500 MPa implied the existence of a narrow window of stability for DNA binding proteins in bacteria. HHP crossing over a certain borderline might trigger an irreversible dissociation of intermolecular forces holding the conformations of DNA interacting proteins, resulting in loss of gene activities supporting cell growth.

Unlike the consistent inactivation of DNA binding activities in different strains exposed to 500 MPa, enzyme activity measurement revealed the resilience of Na^+/K^+-ATPase in *V. parahaemolyticus* under this HHP as raising pressure from 300 to 500 MPa was unable to further repress enzyme activity at all. Increasing HHP from 300 to 500 MPa also failed to induce a pressure-dependent inhibition of catalase activity in *S. Typhimurium*. Thus, the susceptibility of Na^+/K^+-ATPase or catalase to increasing HHP level varied among different bacterial strains.

Gram-negative bacteria are generally more sensitive to pressure than Gram-positive cells [14], yet application of HHP at 500 MPa on Gram-negative *V. parahaemolyticus* and *S. Typhimurium* and Gram-positive *L. monocytogenes* all dramatically suppressed their normal and distorted DNA binding protein activities. Hence, normal and distorted DNA binding proteins are targets of HHP independent of bacterial cell wall structure. Due to the good correlation between HHP-induced destruction of DNA binding capacity and the inability of bacterial cells to resume their growth in phosphate buffer and seafoods treated with the same level of HHP, inactivation of normal and distorted DNA binding protein activities is a potential indicator to discriminate between HHP-induced sublethal and lethal effects on foodborne pathogens.

5. Conclusion

EMSA using a duplex oligonucleotide with a normal or a 6-4PP-twisted helical structure as the binding target detected the presence of normal and distorted DNA binding proteins in the extracts of Gram-positive *L. monocytogenes* and Gram-negative *S. Typhimurium* and *V. parahaemolyticus* cultured under 0.1 MPa. Distorted DNA binding activities in all three strains were weakly inhibited by HHP at 300 MPa. Both normal and distorted DNA binding activities in all three strains were either eliminated or dramatically suppressed to almost undetectable levels after increasing HHP from 300 to 500 MPa, demonstrating the high sensitivity of DNA binding factors to increasing HHP. Unlike the regular responses of DNA binding molecules to HHP, the susceptibility of Na^+/K^+-ATPase or catalase to increasing HHP varied among different strains. Since our previous findings on the absence of bacterial growth in phosphate buffer and seafoods processed with 500 MPa HHP matched very well with the loss of normal and distorted DNA binding protein activities as shown in this study, inactivation of DNA binding activities, very likely a result of irreversible protein damage, is a potential indicator of HHP-induced lethal effects on foodborne bacteria.

Availability of Data and Materials

The data that support the findings shown in this study are available from the corresponding author upon reasonable request.

Author Contributions

YCL performed the research, analyzed the data and acquired funding. PCW optimized the methods, performed the research and analyzed the data. CMW analyzed the data and conducted project administration. TH designed the experiments, validated the data and wrote the original draft. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JFSFQ48851.

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